Transient kinetic studies of *Neurospora crassa* cytochrome *c* oxidase

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The reaction of *Neurospora crassa* cytochrome *c* oxidase with CO was studied by flash-photolysis and rapid-mixing experiments, leading to the determination of the association and dissociation rate constants ($7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 0.02 s^{-1} respectively). Pre-steady-state kinetic investigations of the catalytic properties of the enzyme showed that under proper conditions *Neurospora* cytochrome *c* oxidase can be 'pulsed', i.e. activated, like the mammalian enzyme. The 'pulsed' species is spectroscopically different from the 'resting' one, and the decay into the 'resting' state is fast ($t_{1/2}$ approx. 3 min).

Cytochrome *c* oxidase isolated from the mitochondria of *Neurospora crassa* has proved to be structurally very similar to the bovine enzyme. Thus both enzymes are assembled from subunits comparable in number and size, and they exist in non-ionic detergent solution in dimeric form (Weiss & Sebald, 1978). A comparison of the steady-state properties of the two enzymes, with native and chemically modified cytochrome *c*, has been carried out by Ferguson-Miller *et al.* (1979), who showed that bovine and *Neurospora* cytochrome *c* oxidases display similar kinetic behaviour. In the present paper we report a number of experiments that were performed to explore further the similarities to and differences from bovine cytochrome *c* oxidase. In particular, we have investigated the pre-steady-state kinetic properties of this enzyme and demonstrated that, similarly to other cytochrome *c* oxidases (Antonini *et al.*, 1977; Wilson *et al.*, 1980; Darley-Usmar *et al.*, 1981), the *Neurospora* enzyme exists in either a 'resting' or a 'pulsed' form, the latter being characterized by having an enhanced activity.

Methods

Neurospora crassa cytochrome *c* oxidase was purified by the method of Weiss & Sebald (1978).

Horse heart cytochrome *c* (grade VI) was purchased from Sigma Chemical Co. All the other reagents were of analytical grade.

Absorption spectra were recorded with a Cary 219 spectrophotometer. Cytochrome *c* oxidase concentrations were calculated by using the following molar

absorption coefficient: $\epsilon_{605}^{\text{red.}} = 42000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (molar concentration of functional unit containing two haem *a* groups).

Stopped-flow experiments were performed in a Durrum–Gibson stopped-flow apparatus, equipped with a 2 cm-light-path cell (dead time approx. 3 ms). Kinetics of CO recombination to the reduced enzyme were measured with the flash-photolysis apparatus described elsewhere (Brunori & Giacometti, 1981).

To monitor the spectroscopic decay from the 'pulsed' to the 'resting' species, cytochrome *c* oxidase was reduced under anaerobiosis with a slight excess of $\text{Na}_2\text{S}_2\text{O}_4$. Spectra in the Soret region were recorded as a function of time after exposure to a large excess of oxygen.

Results and discussion

Kinetics of the reaction of Neurospora cytochrome c oxidase with CO

The kinetics of the reaction with CO were investigated by flash photolysis. The time course of recombination after photo-dissociation was measured under pure CO in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ for samples from two different preparations of *Neurospora* cytochrome *c* oxidase. The second-order rate constant was found to be $7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 20°C at pH 7 (50 mM-Tris/acetate buffer containing 0.05% Triton X-100), which is very close to the value reported for the bovine enzyme ($8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) (Gibson & Greenwood, 1963). The kinetic difference spectrum in the Soret region displays an isosbestic point at 436 nm, a peak at 445 nm and a trough at 428 nm. Stopped-flow

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Fig. 1. Time course observed on mixing reduced *Neurospora* cytochrome *c* oxidase with oxygen in the presence of reduced cytochrome *c*

●, Data for 'pulsed' cytochrome *c* oxidase; ○, data for 'resting' cytochrome *c* oxidase. Reactant concentrations after mixing were: cytochrome *c* oxidase, $2.5\mu\text{M}$ (functional unit); cytochrome *c*, $11\mu\text{M}$; ascorbate, 5 mM; *NNN'**N'*-tetramethylphenylenediamine, 0.3 mM. The buffer was 100 mM-sodium phosphate, pH 7.0, containing 0.5% Triton X-100, and the temperature was 25°C . The arrow indicates the direction of absorbance change for cytochrome *c* reduction. A 0.1 unit change in A_{550} corresponds to 25% of haem oxidation.

Fig. 2. Spectroscopic decay from 'pulsed' to 'resting' *Neurospora* cytochrome *c* oxidase

The Figure shows the time course of the spectroscopic changes observed approx. 1 min after exposure of reduced *Neurospora* cytochrome *c* oxidase to O_2 . The fractional time course is obtained from $A_{440} - A_{420}$. The temperature was 20°C for ○ and ●, and 10°C for ▲.

experiments in which the carbonmonoxy derivative of the enzyme was mixed with oxygen-containing buffer yielded kinetic progress curves similar to those reported for the bovine enzyme (Gibson & Greenwood, 1964). The slower component of these curves gave a CO dissociation rate constant of 0.02s^{-1} , close to the value for the mammalian enzyme. Thus the reaction of *Neurospora* cytochrome *c* oxidase with CO is very similar to that characteristic of other cytochrome *c* oxidases previously investigated.

Kinetics of the oxidation of reduced cytochrome *c* by *Neurospora* cytochrome *c* oxidase

The kinetics of the oxidation of reduced cytochrome *c* in air were measured in 50 mM-

Tris/acetate buffer, pH 7, containing 0.05% Triton X-100 at 25°C . The reaction was followed at 550 nm and 605 nm in a stopped-flow instrument, at different concentrations of cytochrome *c* and at constant cytochrome *c* oxidase concentration ($1\mu\text{M}$ functional unit after mixing). Under these conditions, no rapid phase, corresponding to direct electron transfer from reduced cytochrome *c* to oxidized cytochrome *a*, was observed, possibly because of the low enzyme concentration and the high speed of the process under these conditions of low ionic strength. The time course of oxidation of horse cytochrome *c* is exponential, as is the case for bovine cytochrome *c* oxidase (Yonetani & Ray, 1965).

From the dependence of the initial rate on cytochrome *c* concentration, the turnover number

calculated from $V_{\max}$ was 100s^{-1} . This value may be compared with a turnover number of 60s^{-1} obtained for the oxidation of horse cytochrome *c* by the bovine enzyme in air in 0.1 M-phosphate buffer pH 7.0 (Yonetani & Ray, 1965).

The effect on the catalytic activity of *Neurospora* cytochrome *c* oxidase of subjecting it to a cycle of complete reduction followed by oxidation by oxygen was studied at pH 7 in a 100 mM-sodium phosphate buffer containing 0.05% Triton X-100. The objective was to determine whether the fungal enzyme could be 'pulsed', i.e. activated, in a similar way to the enzyme isolated from mammals and fish. The experiments were performed by comparing two parallel conditions, i.e. by mixing either (i) oxidized cytochrome *c* oxidase in air with reduced cytochrome *c* in the presence of sodium ascorbate and *NNN'*-tetramethylphenylenediamine ('resting'-oxidase experiment) or (ii) reduced cytochrome *c*, sodium ascorbate and *NNN'*-tetramethylphenylenediamine with O_2 -equilibrated buffer ('pulsed'-oxidase experiment) (see Antonini *et al.*, 1977). The reaction was monitored in a stopped-flow apparatus at 605 nm and 550 nm; the time courses of the absorbance changes at 550 nm are reported in Fig. 1 for the two experimental conditions.

It is evident that *Neurospora* cytochrome *c* oxidase may be 'pulsed', leading to a more active state. Thus with the 'pulsed' enzyme the approach to the steady state is faster, the steady-state phase (Fig. 1) is flat and shorter, and the redox level is more clearly shifted towards oxidized cytochrome *c*. These phenomena are both very similar to the effects described for bovine cytochrome *c* oxidase (Brunori *et al.*, 1979), and clearly show that with *Neurospora* cytochrome *c* oxidase activation from the 'resting' to the 'pulsed' form also occurs during turnover. Quantitative analysis of the results is not yet possible, but it may be advanced that the transition from 'resting' reduced to 'pulsed' reduced ($R_r \rightarrow P_r$) (see the model proposed by Wilson *et al.*, 1981) occurs within much less than 1 s.

To extend the comparison, we have measured the decay from 'pulsed' oxidized to 'resting' oxidized ($P_o \rightarrow R_o$), by using the spectral difference previously assigned in the case of the bovine enzyme (Colosimo *et al.*, 1981). Fig. 2 reports the time course of the spectral decay, and shows: (i) that the time course is not a single phase (both at 20°C and at 10°C); (ii) that the faster kinetic component, which accounts for approx. 70% of the total change at 20°C, has a half-time of approx. 3 min.

General conclusion

The kinetic investigation reported in the present paper shows that the functional properties of

Neurospora cytochrome *c* oxidase are indeed very similar to those of the bovine enzyme. These new data on transient kinetics are in very good agreement with previous steady-state results and with expectations based on the structural and spectral properties of the enzyme as compared with bovine cytochrome *c* oxidase. This body of results supports the conclusion that evolutionary changes in mitochondrial cytochrome *c* oxidases have been of a minor nature, the complex being highly conserved in its basic functional features.

Particularly important is our observation that *Neurospora* cytochrome *c* oxidase can also be 'pulsed', i.e. activated, and that the differences in kinetic and spectral properties between 'pulsed' and 'resting' *Neurospora* cytochrome *c* oxidase are similar to those previously reported for bovine and shark cytochrome *c* oxidases. This contributes to indicate the general applicability of the two-state model proposed to describe the functional properties of cytochrome *c* oxidase (Colosimo *et al.*, 1981).

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